

# Are Altered $\text{pH}_i$ and Membrane Potential in hu MDR 1 Transfectants Sufficient to Cause MDR Protein-mediated Multidrug Resistance?

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**ABSTRACT** Multidrug resistance (MDR) mediated by overexpression of the MDR protein (P-glycoprotein) has been associated with intracellular alkalinization, membrane depolarization, and other cellular alterations. However, virtually all MDR cell lines studied in detail have been created via protocols that involve growth on chemotherapeutic drugs, which can alter cells in many ways. Thus it is not clear which phenotypic alterations are explicitly due to MDR protein overexpression alone. To more precisely define the MDR phenotype mediated by hu MDR 1 protein, we co-transfected hu MDR 1 cDNA and a neomycin resistance marker into LR73 Chinese hamster ovary fibroblasts and selected stable G418 (geneticin) resistant transfectants. Several clones expressing different levels of hu MDR 1 protein were isolated. Unlike previous work with hu MDR 1 transfectants, the clones were not further selected with, or maintained on, chemotherapeutic drugs. These clones were analyzed for chemotherapeutic drug resistance, intracellular pH ( $\text{pH}_i$ ), membrane electrical potential ( $V_m$ ), and stability of MDR 1 protein overexpression. LR73/hu MDR 1 clones exhibit elevated  $\text{pH}_i$  and are depolarized, consistent with previous work with LR73/mu MDR 1 transfectants (Luz, J.G. L.Y. Wei, S. Basu, and P.D. Roepe. 1994. *Biochemistry*. 33:7239–7249). The extent of these perturbations is related to the level of hu MDR 1 protein that is expressed. Cytotoxicity experiments with untransfected LR73 cells with elevated  $\text{pH}_i$  due to manipulating percent  $\text{CO}_2$  show that the  $\text{pH}_i$  perturbations in the MDR 1 clones can account for much of the measured drug resistance. Membrane depolarization in the absence of MDR protein expression is also found to confer mild drug resistance, and we find that the  $\text{pH}_i$  and  $V_m$  changes can conceivably account for the altered drug accumulation measured for representative clones. These data indicate that the MDR phenotype unequivocally mediated by MDR 1 protein overexpression alone can be fully explained by the perturbations in  $V_m$  and  $\text{pH}_i$  that accompany this overexpression. In addition, MDR mediated by MDR protein overexpression alone differs significantly from that observed for MDR cell lines expressing similar levels of MDR protein but also exposed to chemotherapeutic drugs.

**KEY WORDS:** P-glycoprotein • multidrug resistance • intracellular pH • altered partitioning

## INTRODUCTION

Elucidation of the molecular mechanism of multidrug resistance (MDR)<sup>1</sup> protein-mediated multidrug resistance is a key goal of cancer pharmacology as well as membrane transport physiology. Recent analysis of MDR protein function (Bornmann and Roepe, 1994; Luckie et al., 1994; Luz et al., 1994; Reutz and Gros, 1994; Hardy et al., 1995; Shapiro and Ling, 1995) and other data (Wadkins and Houghton, 1993; Mayer et al., 1985*a, b*; Mayer et al., 1986; Praet et al., 1993; Wadkins and Houghton, 1995; Wei et al., 1995; Wadkins and Roepe, 1996) suggest that the mechanism for altered

drug retention and resistance in MDR cells is more complex than initially thought (Danø, 1973). First, a distinction should be made between phenotypic features that are unequivocally mediated by overexpression of MDR protein and those that may be mediated by other events due to exposing cells to chemotherapeutic drugs (Roepe, 1995). With a few exceptions (Guild et al., 1988; Devault and Gros, 1990; Valverde et al., 1992; Luz et al., 1994) analysis of MDR protein function has been done with cells selected or maintained on potent chemotherapeutic drug(s). These drugs have a variety of complex effects, thus the precise description of the MDR protein-mediated MDR phenotype remains vague. This greatly clouds discussions of MDR protein function. For example, most model MDR cell lines exhibit  $\geq 10^2$ -fold levels of drug resistance, but MDR protein overexpression per se has not yet been associated with particularly high ( $>10$ -fold) levels of drug resistance (Guild et al., 1988; Devault and Gros, 1990). Thus, most studies (including analysis of MDR protein mutants) have likely examined behavior that is at least in part due to drug exposure rather than MDR protein overexpression. It is critical that MDR protein

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<sup>1</sup>Abbreviations used in this paper: ABC, ATP-binding cassette; AE, anion exchange or  $\text{Cl}^-/\text{HCO}_3^-$  exchange; CFTR, cystic fibrosis transmembrane conductance regulator; col, colchicine; dox, doxorubicin (adriamycin); G418, geneticin; MDR, multidrug resistance;  $\text{pH}_i$ , intracellular pH; SCP, single-cell photometry;  $V_i$ , intracellular volume;  $V_m$ , plasma membrane electrical potential; vncr, vincristine.

be studied using "pure" transfectants that have not been exposed to chemotherapeutic drugs before analysis.

A vigorously championed model for MDR protein is that it acts as a drug pump, coupling hydrolysis of adenosine 5'-triphosphate (ATP) to direct movement of drug out of a cell against a concentration gradient (Gottesman and Pastan, 1993; Sharom et al., 1993; Ruetz and Gros, 1994; Shapiro and Ling, 1995). However, verification of predicted thermodynamic and kinetic features of putative drug pumping has been difficult to obtain (Roepe, 1992; Bornmann and Roepe, 1994; Shapiro and Ling, 1995; Roepe et al., 1996). This has led to (a) more involved models such as the "flippase" (Higgins and Gottesman, 1992) or "vacuum cleaner" (Gottesman and Pastan, 1993) models, which attempt to account for the observed unusual thermodynamic and kinetic features or (b) alternative "indirect" models for how overexpression of MDR protein could promote resistance to hundreds of structurally divergent compounds without invoking direct active transport (e.g., the "altered partitioning model," see Roepe, 1995; Wadkins and Roepe, 1996). A third model is the "dual function" model (Gill et al., 1992) in which the protein alternates between  $\text{Cl}^-$  channel or channel regulator and drug pump conformations; a fourth is the ATP channel hypothesis (Abraham et al., 1993). The ATP channel model implies that drug resistance may be due to changes in  $\text{pH}_i$  and/or  $V_m$  triggered by increased extracellular ATP (Huang et al., 1992; El-Moatassim et al., 1992), presumably via a purinergic receptor pathway (Al-Awqati, 1995).

Since MDR cells exhibit decreased accumulation of many dozens of structurally divergent compounds, any permutation of a pump model envisions that MDR protein is an extraordinary enzyme that violates the law of enzyme specificity. Also, recent studies (Sharom et al., 1993; Ruetz and Gros, 1994; Schlemmer and Sirotiak, 1994) that measure putative MDR protein-catalyzed drug pumping yield very slow estimated turnover at reasonable concentrations of drug,<sup>2</sup> as well as very unusual and highly variable ATP hydrolysis:drug translocation stoichiometries.<sup>3</sup> Because of these and other difficulties

summarized elsewhere (Roepe, 1995; Roepe et al., 1996; Wadkins and Roepe, 1996), it is difficult to visualize how this measured translocation activity (be it pumping, "flipping," or "vacuuming") could compete with passive diffusion to lower drug accumulation under initial rate conditions as is typically observed (see Hammond et al., 1989; Stein et al., 1994; Robinson and Roepe, 1996), or how the putative pump couples ATP hydrolysis to drug translocation. Demant et al. (1990) estimated the kinetic requirements of a drug pump necessary to decrease accumulation of drugs in MDR cells, and these estimates are orders of magnitude higher than the putative drug pumping measured with vesicles (Ruetz and Gros, 1994; Schlemmer and Sirotiak, 1994) or proteoliposomes (Sharom et al., 1993; Shapiro and Ling, 1995). Thus, along with the lack of specificity, significant kinetic and thermodynamic "dilemma" are revealed when attempting to reconcile the pump hypothesis with available data and theory.

Other data suggest the MDR phenotype is, at least in part, due to  $\text{pH}_i$  and/or membrane potential ( $V_m$ ) changes that accompany MDR protein overexpression (Roepe, 1995). Translocation and retention of chemotherapeutic drugs are influenced by small perturbations in  $\text{pH}_i$  or  $V_m$  (Mayer et al., 1985*a, b*; Mayer 1986; Praet et al., 1993; Simon et al., 1994; Wadkins and Roepe, 1996) and perturbations of similar, if not greater magnitude typically occur in MDR cells (Roepe, 1995). In contrast to these data, one study reports no change in  $\text{pH}_i$  for a hu MDR 1 transfectant further selected with adriamycin (Altenberg et al., 1993). However, this study did not examine  $V_m$ , and it has been shown (Wei et al., 1995) that depolarization is sufficient to confer MDR. Also, the cells studied by Altenberg et al. were not analyzed for MDR protein expression by immunoblot. This caveat is important, since MDR protein overexpression can be unstable in transfectants (Luz et al., 1994, and RESULTS) and drug resistance per se in drug selected cells is no guarantee of a MDR protein-mediated MDR phenotype. Again, drug selection of model cell lines complicates analysis.

The altered partitioning model envisions that these  $\text{pH}_i$  and  $V_m$  perturbations alter diffusion/retention of chemotherapeutic drugs via several overlapping mechanisms (Roepe, 1995; Wadkins and Roepe, 1996). It is unknown how these perturbations occur, but there are several possibilities. For example, although there are also conflicting data (Ehring et al., 1994), a variety of reports describe anomalous  $\text{Cl}^-$  transport in MDR cells

<sup>2</sup>The vast majority of interpretable drug transport measurements using cells, vesicles or proteoliposomes have analyzed transport of  $10^{-9}$  to  $10^{-6}$  M levels of drug. At concentrations above several  $\mu\text{M}$ , drugs in the "MDR spectrum" form aggregates, and/or exhibit other complex behavior.  $10^{-5}$  to  $10^{-4}$  M concentrations of these drugs and other agents believed to be "pumped" by MDR protein can cause significant disruption of lipid structure (see Wadkins and Houghton, 1993) that greatly complicates analysis of transport. It is difficult to interpret the significance of drug translocation kinetics at concentrations greater than several  $\mu\text{M}$ .

<sup>3</sup>The coupling principle states that a defined stoichiometry of energy consumption:substrate translocation (in this case, ATP hydrolysis:drug translocation) exists for any pump. For ATP-coupled pumps,

these stoichiometries are typically between 1:1–1:3. Examination of recent drug transport data reveals that extremely high and widely different ATP hydrolysis:drug translocation stoichiometries can be calculated in the specific case of MDR protein-mediated drug translocation (see Roepe, 1995; Roepe et al., 1996).

(Valverde et al., 1992; Hardy et al., 1995) and are thus consistent with the altered partitioning model, since  $V_m$  could be perturbed by changes in  $Cl^-$  permeability. Chemotherapeutic drug selection of cells analyzed for  $Cl^-$  transport may be responsible for discrepancies in the reported data. Another suggestion (Al-Awqati, 1995; Schwiebert et al., 1995) is that ABC (ATP-binding cassette) transporters indirectly modulate ion conductances (which would then influence  $V_m$  and/or  $pH_i$ ) through an autocrine mechanism dependent upon ATP export. However, this model has recently been strongly challenged (Li et al., 1996; Reddy et al., 1996) via studies with purified CFTR protein. Other analysis (Roepe et al., 1993; Luz et al., 1994) has revealed paradoxical phenomena such as reduced AE (anion exchange or  $Cl^-/HCO_3^-$  exchange) activity concomitant with increased AE expression in MDR cells. If some version of an altered partitioning model is entertained, it is important to deduce which  $pH_i/V_m$  alterations are unequivocally due to MDR protein overexpression, and also how they occur.

In devising additional experiments, it is critical to realize that most model MDR cell lines have been created via protocols that include growth on chemotherapeutic drugs, which induce many phenomena. Although MDR transfectants are in theory an improvement, almost all of these have also been grown in the presence of chemotherapeutic drug (Choi et al., 1988; Lincke et al., 1990; Gros et al., 1991; Currier et al., 1992; Loo and Clarke, 1993; Stein et al., 1994; Beaudet and Gros, 1995). To resolve remaining questions, new experimental systems are required, and detailed biophysical analysis of these is essential.

Therefore, we have created hu MDR 1 transfectants expressing variable levels of MDR protein without exposing the cells to chemotherapeutic drugs. We have analyzed their  $pH_i$ ,  $V_m$ , drug resistance, and other traits. The data better define the MDR protein-mediated MDR phenotype and suggest that measured  $V_m$  and  $pH_i$  changes are sufficient to promote drug resistance and altered drug accumulation unequivocally due to MDR protein overexpression alone.

## MATERIALS AND METHODS

### Materials

2',7'-bis (carboxyethyl)-5, 6-carboxyfluorescein (BCECF), the oxacarboxyanines 3,3' diethyloxacarboxyanine iodide ( $diOC_2(3)$ ), 3,3' dipentyloxacarboxyanine iodide ( $DiOC_5(3)$ ), and 3,3' diheptyloxacarboxyanine iodide ( $DiOC_7(3)$ ), nigericin, valinomycin and the dialkylaminonaphthalene pyridinium styryl dye di-4-ANEPPS were purchased from Molecular Probes (Eugene, OR) and used without further purification. 4-acetamido-4'-isothiocyanato stilbene-2,2'-disulfonic acid (SITS), colchicine, and verapamil were from Sigma Chemical Co. (St. Louis, MO), doxorubicin, vincristine, and cyclosporin A were from the Memorial Sloan-Kettering phar-

macy (New York, NY), and G418 was from Life Technologies (Grand Island, NY). All other chemicals were reagent grade or better, purchased from commercial sources, and used without further purification.

### Tissue Culture

Cell lines stably transfected with hu MDR 1 cDNA were created by transfecting a mixture of two plasmid constructs, one harboring a neomycin resistance gene (pSVDNAneo obtained from Invitrogen Corp., San Diego, CA), and the other the hu MDR 1 cDNA cloned behind a cytomegaloviral promoter (plasmid pMDR1), at a 1:10 molar ratio via the  $CaPO_4$  precipitation method. The open reading frame of MDR cDNA has been sequenced and, with the exception of a silent Ser 180 point mutation, no differences relative to that published (Chen et al., 1986) have been found. Mass populations of LR73 transfectants were selected with 500  $\mu g$  /ml active G418; a concentration that did not allow negative control transfectants (pMDR1 without pSVDNAneo) to survive. Over 100 G418 resistant colonies were then replica-plated in 24-well plates. One well of a pair was grown in the presence of 100 ng/ml doxorubicin. The second well was grown in the presence of G418 without any chemotherapeutic drug. From an initial screen of over 100 G418 resistant LR73 clones, 5 that survived doxorubicin selection were found to express MDR protein (see below). An additional 3 overexpressors were found among 35 additional colonies from a second transfection. Thus, we find that a low frequency (<10%) of G418 resistant clones apparently overexpress significant MDR 1 protein. After screening, cells from the sister "G418-only" wells were propagated in the presence of G418 only and screened for MDR protein expression via western blot (see RESULTS). These clones were, with one exception (see below), never exposed to any chemotherapeutic drug before analysis. Cells analyzed for  $V_m$  or  $pH_i$  (see below) were propagated in the absence of G418 for not more than 10 d. These conditions did not measurably reduce levels of MDR protein. Stock cultures of the stable transfectants were frozen in liquid nitrogen and resuscitated as needed.

In one instance, hu MDR 1 clone #27 was exposed to chemotherapeutic drug before analysis (described in the legend to Table II) for the specific purpose of determining how this exposure effected the drug resistance profile (see RESULTS).

LR73 transfectants were grown at 37°C in a 5%  $CO_2$  atmosphere in Dulbecco's modified Eagle's (DME) medium supplemented with 10% FCS, 200 U/ml of penicillin, and 100  $\mu g$ /ml of streptomycin. For mass cell population  $V_m$  measurements or Western blot analysis (see below), they were harvested by trypsinization, washed, and gently resuspended in fresh media.

For single-cell photometry analysis of  $pH_i$  and  $V_m$ , the cells were grown as above but on glass coverslips (18  $mm^2/0.11$  mm thick; Corning Glassworks, Corning, NY) immobilized in standard tissue culture plates with a dab of autoclaved silicon vacuum grease (Dow-Corning, Midland, MI). Coverslips were kept in media at 37°C and 5%  $CO_2$  until immediately before mounting on a perfusion chamber (see single-cell photometry below and Luz et al., 1994; Wei et al., 1995).

### Drug Resistance

Resistance was assayed by colony formation as described (Wei et al., 1995). Surviving colonies >50 cells were scored visually. Plating efficiency at each drug concentration was analyzed in duplicate. Resistance was also assayed by growth inhibition as described (Wei and Roepe, 1994).

## Drug Accumulation

Measurement of anthracycline, vinca alkaloid, or colchicine accumulation was essentially as described (Wei and Roepe, 1994) with some modifications. We assayed  $^3\text{H}$ -daunorubicin,  $^3\text{H}$ -vinblastine, or  $^3\text{H}$ -colchicine (New England Nuclear, Boston, MA) accumulation by plating cells in six well plates ( $5.0 \times 10^4$  cells/well initial density), incubating the plates in normal DME medium at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  (see tissue culture, above) for 3 d until they reached 75% confluency, and then exposing them to the  $^3\text{H}$  analogues (50 nM, 200  $\mu\text{Ci}/\text{mM}$  for  $^3\text{H}$ -daunorubicin; 50 nM, 75  $\mu\text{Ci}/\text{mM}$  for  $^3\text{H}$ -vinblastine; 50 nM, 200  $\mu\text{Ci}/\text{mM}$  for  $^3\text{H}$ -colchicine) for exactly 60 min. Drug-containing medium was then aspirated; cells were washed once with cold PBS to remove non-internalized drug, and cells then were harvested by trypsinization. Trapped radioactivity was measured by liquid scintillation spectrometry. Nonspecific drug association (zero-time uptake) was measured by incubating an identical number of cells in medium pre-chilled to  $4^\circ\text{C}$ . Cell number was counted using a hemocytometer, and aliquots of the collected cells were assayed for protein content by the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA). Drug accumulation was expressed as both picomoles drug/microgram total cell protein and nM internalized drug (see Table I for cell volumes). Protein content per cell was not dramatically different for the different cell lines. Accumulation was measured in triplicate for each cell line in each accumulation assay, and the results from three such assays were averaged to yield the data shown in results (SE <10%). Data is expressed as a percentage of accumulation, with values for LR73/neo controls defined as 100% accumulation (see Fig. 11 in Results).

## Pulse Elevation of $\text{pH}_i$ and Short Exposure Cytotoxicity Assays

We wished to assay chemotherapeutic drug resistance for cells with alkaline  $\text{pH}_i$  that did not express measurable MDR protein. Thus, to elevate  $\text{pH}_i$  of LR73 cells, they were plated at a density of  $10^5/100 \text{ cm}^2$  and grown overnight at 5%  $\text{CO}_2$  in normal DME medium initially harboring 44 mM  $\text{HCO}_3^-$ . They were then shifted to a 10%  $\text{CO}_2$  atmosphere, which causes acidification of the medium (see Wei and Roepe, 1994). Milder acidification of  $\text{pH}_i$  accompanies this " $\text{CO}_2$  pulse" (see RESULTS). Within 36–48 h the  $\text{pH}_i$  acidification plateaus, and the cells then nearly recover  $\text{pH}_i$  (see RESULTS). If the conditioned cells are then placed back in a 5%  $\text{CO}_2$  atmosphere in the presence of fresh pre-equilibrated media,  $\text{pH}_i$  "overshoots" and is  $\sim 0.15$ – $0.22$  units alkaline, relative to normal resting  $\text{pH}_i$ , for  $\sim 12$ – $24$  h (see RESULTS). After this time, the cells "re-adapt" and return to normal  $\text{pH}_i$ .

Because the cells are alkaline for only a short time, we could not measure resistance for alkaline cells via colony formation or growth inhibition assays, since these require days of growth in the presence of the drugs. Therefore, we devised "short pulse" cytotoxicity assays. In these experiments, any drug resistance may be underestimated, since cytotoxic actions of chemotherapeutic drugs are not necessarily analogous to their growth inhibitory actions. MDR protein may confer resistance to both.

Cells were harvested by trypsinization, washed in fresh media pre-equilibrated in a 5%  $\text{CO}_2$  atmosphere, and  $1.0 \times 10^5$  aliquots were resuspended in 1 ml of fresh 5%  $\text{CO}_2$ -equilibrated media harboring different concentrations of drug (see RESULTS). The cell suspensions were tumbled in eppendorf tubes in a 5%  $\text{CO}_2$  atmosphere, and the cells were collected, washed in fresh 5%  $\text{CO}_2$ -equilibrated media, and then  $2 \times 10^4$  aliquots were plated in each of four wells of a 96-well plate. The plates were grown for 3 d in an atmosphere of 5%  $\text{CO}_2$ , and then stained with crystal violet, after which the cells were solubilized, and dye was quantitated with an ELISA reader (see Wei and Roepe, 1994). In separate experiments we verified that the protein content/cell was the same

for the different cell preparations. Four determinations at each drug concentration were then averaged, and the results from four such assays (16 determinations in all) were averaged (see RESULTS).

Similar experiments were also performed at different  $[\text{K}^+]_o$  to test the effect of altered  $V_m$  (see RESULTS) on drug resistance. Cells were cultured at 5%  $\text{CO}_2$ , washed in DME media harboring either 127 mM  $\text{Na}^+/15$  mM  $\text{K}^+$  or 127 mM  $\text{K}^+/15$  mM  $\text{Na}^+$  and then tumbled (as above) in the same media plus varied concentrations of chemotherapeutic drugs. Cells were then washed in fresh normal media, plated, grown for 3 d, and stained as above.

## Western Blotting

Western blots were performed using the MDR protein-specific mAb C219 and the enhanced chemiluminescence detection system (Amersham Corp., Arlington Heights, IL) essentially as described (Wei and Roepe, 1994) but with some modifications in how cell lysates were prepared. Cells were harvested from a  $100\text{-cm}^2$  plate after growth at 5%  $\text{CO}_2$  to near confluency, washed with PBS, pH 7.3, and stored at  $-80^\circ\text{C}$ . Cells were thawed and lysed in  $\sim 200 \mu\text{l}$  cracking buffer (10% glycerol, 1% Triton X-100, 1 mM PMSF, 10  $\mu\text{g}/\text{ml}$  leupeptin, 20 mM HEPES). They were then vortexed, incubated at  $4^\circ\text{C}$  for 15 min, and spun in a microcentrifuge (12,000 rpm) at  $4^\circ\text{C}$  for 5 min. The supernatant was removed, and 1  $\mu\text{l}$  was assayed with the Bio-Rad protein assay, with BSA as a standard. 100  $\mu\text{g}$  of cellular protein was then resolved by sodium dodecyl sulfate-PAGE and blotted to nitrocellulose overnight at 40 mA constant current (Wei and Roepe, 1994). After Western blots were performed as described (Wei and Roepe, 1994), the autoradiogram was scanned with a 300 dpi scanner (Stratagene Inc., La Jolla, CA). The intensity of bands of interest were normalized to background emission from the blot and quantitated with Stratagene densitometry software using an AST computer.

## Fluorescence Spectroscopy

Fluorescence spectra were obtained with a Photon Technology International (South Brunswick, NJ) fluorometer interfaced to a Dell/433L computer. Sample cuvettes were jacketed within an aluminum holder, and temperature was controlled by a circulating water bath. Cell suspensions were rapidly mixed with a magnetic stirrer situated beneath the cuvette. Excitation/emission wavelengths and the other parameters of various experiments may be found in the individual figure legends.

## Single-Cell Photometry (SCP) and Measurement of $\text{pH}_i$

As described (Luz et al., 1994; Wei et al., 1995), we have constructed a single-cell photometry apparatus using a Nikon diaphot microscope and a Photon Technology alphascan fluorometer. For BCECF experiments a 510-nm dichroic and a 530-nm band-pass filter was positioned beneath the stage, and the excitation monochromator was flipped between 439 and 490 nm by the computer (see Wei et al., 1995). For carbocyanine experiments, we used a 510 dichroic and a 495 high pass filter under the microscope stage, and the excitation monochromator was fixed at 465 nm.

Cells were grown on sterile glass coverslips as described above and used  $>2$  but  $<6$  d after plating. Coverslips were incubated with BCECF-AM in DME media for 30 min before mounting in a perfusion chamber, which provided unilamellar perfusate flow (Wei et al., 1995). After mounting, cells were continuously perfused with Hank's balanced salt solution (HBSS: 118 mM NaCl, 5 mM KCl, 10 mM glucose, 24.2 mM  $\text{NaHCO}_3$ , 1.3 mM  $\text{CaCl}_2$ , 0.5 mM  $\text{MgCl}_2$ , 0.5 mM  $\text{Na}_2\text{HPO}_4$ , 0.6 mM  $\text{KH}_2\text{PO}_4$ ; pH 7.33) equili-

brated with 5% CO<sub>2</sub> and to 37°C. Flow rate was 5 ml/min, and perfusate volume in the perfusion chamber was ~100 µl, so buffer over the cells was exchanged within 1–2 s. Uniform BCECF staining was verified by monitoring 490-nm excitation intensity; we did not observe significant differences in BCECF loading for MDR vs. control transfectants (see RESULTS). All buffers harboring HCO<sub>3</sub><sup>-</sup> were continuously purged with 5% CO<sub>2</sub> (balance air), and pH<sub>o</sub> was monitored with an electrode. Exposure to excitation was limited to the time of data collection to limit photobleaching.

Steady-state pH<sub>i</sub> was computed by averaging 439/490 nm excitation for 12–20 individual cells (data collected 1 cell at a time) perfused with HBSS before the same coverslip was exposed to calibration solution containing nigericin (100 mM KCl, 40 mM NaCl, 20 mM HEPES, 1.5 µM nigericin) equilibrated to various pH (see Fig. 3) and then inserting the averaged value into a quadratic fit to the calibration solution standard curve. Calibration curves were obtained using a “single cell” version of the K<sup>+</sup>/nigericin titration approach of Thomas and colleagues (1979) as described (see RESULTS and Luz et al., 1994), and they were generated for every coverslip used to measure steady-state pH<sub>i</sub> under physiologic conditions.

### Measurement of Electrical Membrane Potential (V<sub>m</sub>)

K<sup>+</sup>/valinomycin null point titration (Laris and Hoffman, 1986), using the fast response ratiometric styryl dye di-4-ANEPPS (Montana et al., 1989), was used to quantitatively estimate V<sub>m</sub> as described in detail previously (Roepe et al., 1993; Luz et al., 1994; Wei et al., 1995). We have recently published the results of a double-blind analysis (Wei et al., 1995) wherein V<sub>m</sub> measured for CFTR overexpressors by electrophysiology techniques (zero current clamp potential recorded within 10 s of establishing whole cell configuration; see Stutts et al., 1993) was compared to V<sub>m</sub> measured by our K<sup>+</sup>/valinomycin titration protocol. Although the values obtained did not agree exactly, significant differences in V<sub>m</sub> between cell lines were easily distinguished by either method, and the general trend in V<sub>m</sub> observed in a series of CFTR clones expressing different levels of functional channel was essentially the same via both methods. Contributions to the measured V<sub>m</sub> from mitochondria and other organelles as well as variability in the concentration of cell-associated dye are less of a concern with di-4-ANEPPS measurements, and since it is a fast response probe, measurements are made in seconds rather than minutes, avoiding altered dye partitioning within the cell during the valinomycin titration (see also Roepe et al., 1993). Thus, any postulated pumping of the dye by MDR protein would not, if it even existed, bias the measurements. We compensate for the low di-4-ANEPPS signal by averaging several traces from multiple titrations at each [K<sup>+</sup>]<sub>o</sub>. As reported previously (Roepe et al., 1993; Luz et al., 1994), [K<sup>+</sup>]<sub>i</sub> does not vary substantially between MDR and sensitive cells, thus an average value of 160 mM was used to calculate V<sub>m</sub>.

To test conclusions from the di-4-ANEPPS experiments, we also measured carbocyanine uptake via single cell photometry. Coverslips were perfused with HBSS, pH 7.30, as described for pH<sub>i</sub> measurements above. We then switched to HBSS perfusate containing 500 nM diOC<sub>2</sub>(3), diOC<sub>5</sub>(3), or diOC<sub>7</sub>(3) and monitored the increase in cellular fluorescence >510 nm (465-nm excitation) while the cells were under constant perfusion. Wavelength-dependent excitation and emission spectra in the presence or absence of cells verified the respective maxima were not appreciably shifted by binding to cells. HBSS/carbocyanine solutions were prepared immediately before perfusion from a 1 mM stock made in anhydrous DMSO and HBSS pre-equilibrated in 5% CO<sub>2</sub> and to 37°C. In some cases, K<sup>+</sup> was substituted for Na<sup>+</sup> in

the HBSS to depolarize cells (see RESULTS). As pointed out in a recent paper by Wadkins and Houghton (1995) and as first described by Hoffman and colleagues (Sims et al., 1974), longer chain length trimethine carbocyanines (diOC<sub>7</sub>(3)-diOC<sub>8</sub>(3)) are less responsive to membrane potential than moderate chain length carbocyanines (e.g., diOC<sub>5</sub>(3)), and in many cell types, decreasing chain length further does not elicit a more V<sub>m</sub>-dependent response and may actually decrease the responsiveness of the carbocyanine to V<sub>m</sub>, at least as measured by changes in dye partitioning upon treatment with valinomycin. Redistribution should be distinguished from initial accumulation when discussing V<sub>m</sub> probes; both “redistribution” upon changing V<sub>m</sub> and initial accumulation are V<sub>m</sub> dependent, and both responses are dye concentration dependent (see Sims et al., 1974; Roepe et al., 1993; Wadkins and Houghton, 1995). Thus, plotting differences in the rate and/or steady state of accumulation of the different probes for “matched” cell lines (e.g., derivatives of the same parental cell line with similar size) is a legitimate complementary estimate of any difference in membrane potential (see RESULTS). For cells with different V<sub>m</sub>, accumulation of longer-chain carbocyanines (relatively non-V<sub>m</sub>-dependent at low concentrations) should be similar, and relative probe accumulation should diverge as chain length approaches 5 carbons (i.e., for V<sub>m</sub>-dependent probes). A series of experiments, although tedious, will correct for nonspecific effects of dye association that are unrelated to V<sub>m</sub>. Accumulation of each carbocyanine was measured for each cell line at least four times using four different coverslips, and the data were averaged (see RESULTS).

### Measurement of [K<sup>+</sup>]<sub>i</sub>

Cell-associated K<sup>+</sup> was measured by flame photometry as described (Iversen, 1976; Roepe et al., 1993). After determining intracellular volume (see below), [K<sup>+</sup>]<sub>i</sub> was calculated.

### Measurement of Intracellular Volume (V<sub>i</sub>)

Total cell volume was calculated after determining the mean particle size of cell suspensions by the single threshold Coulter method (Kachel, 1990; Roepe et al., 1993). Intracellular water volume was determined by ratioing <sup>14</sup>C-inulin vs. <sup>3</sup>H<sub>2</sub>O dpm as described (Rottenberg, 1979; Roepe et al., 1993).

## RESULTS

### Characterization of hu MDR 1 Expression and pH<sub>i</sub> for Transfectant Clones

Table I summarizes characteristics of a series of 6 LR73 pSVDNNeo/pMDR1 transfectants created and isolated as described in MATERIALS AND METHODS. Also included for comparison purposes is similar data for the cell line EX4N7 (Gros et al., 1991), which is LR73 transfected with mu MDR 1 and a neo<sup>r</sup> marker and selected with G418 only, as well as data for control transfectants (LR73/neo) which do not express MDR 1 protein but were otherwise treated identically relative to the hu MDR 1 clones.

Fig. 1 A presents representative data from Western blot analysis of MDR 1 protein expression in these transfectants and shows that we isolated clones with different levels of MDR protein without subjecting the cells to different levels of chemotherapeutic drug selec-

TABLE I  
Characteristics of True hu MDR1 Transfectants

Cell line	Relative MDR protein	Size ( $\mu\text{m}$ )	$\text{pH}_i$	$V_m$ ( $\pm \leq 4$ mV)	Fold resistance to		
					Dox	Vncr	Col
LR73	0	12.04 $\pm$ 0.27	7.14 $\pm$ 0.03	45	—	—	—
LR73/neo	0	11.91 $\pm$ 0.19	7.16 $\pm$ 0.03	46	—	—	—
LR73/21	1	11.87 $\pm$ 0.74	7.38 $\pm$ 0.06	38	1.2	2.1	1.5
LR73/24	11.7	11.91 $\pm$ 0.11	7.48 $\pm$ 0.04	24	2.8	10.2	4.1
LR73/27	9.7	11.86 $\pm$ 0.26	7.51 $\pm$ 0.05	26	2.7	9.5	3.2
LR73/71	2.7	11.72 $\pm$ 0.06	7.42 $\pm$ 0.04	29	1.6	3.4	3.1
LR73/88	2.1	11.85 $\pm$ 0.21	7.40 $\pm$ 0.03	31	1.9	2.6	3.3
LR73/95	2.8	11.57 $\pm$ 0.17	7.37 $\pm$ 0.05	35	2.1	3.4	1.8
EX4N7 (LR73/mu MDR 1)	5.1	N.D.	7.31 $\pm$ 0.04	23	3.6	9.2	4.5

Relative MDR protein (via densitometry), size  $\pm$  SD (by the single threshold Coulter method),  $\text{pH}_i \pm$  SD (by SCP),  $V_m \pm$  SE (by  $\text{K}^+$ /val titration with di-4-ANEPPS; see Roepe et al., 1993; Luz et al., 1994), and drug resistance (by colony formation; see Wei et al., 1995) for LR73-derived hu MDR 1 transfectants used in the present work, as well as parental LR73 cells and a negative control transfectant also selected with G418. Relative MDR protein is the average of three densitometric determinations from three Western blots; note clone #21 is assigned a value of 1. Fold-resistance was determined by either growth inhibition or colony formation assays and ratioing  $\text{IC}_{50}$ s that were calculated by exponential fits to the data ( $R^2 > 0.92$  in each case, see Wei et al., 1995 and MATERIALS AND METHODS). Data shown is the average of two determinations (Wei et al., 1995). N.D. denotes not done.

tion. Overexpression of mu MDR 1 protein may be unstable in some transfectants; that is, EX4N7 cells cultured for several months in the presence of G418 eventually lose mu MDR 1 protein (Luz et al., 1994). As shown in Fig. 1 B, we again find this to be the case for some, but not all, hu MDR 1 transfectants. As some clones are passed in the presence of G418, overexpression of MDR protein decreases, and eventually disappears after 14–21 passages. Thus, most subsequent work was performed with cell lines exhibiting stable overexpression (e.g., clones #24, #27, and #95), and overexpression levels were routinely checked every 6–10 passages. We do not find that high level expressors are necessarily less stable with regard to expression over time, in fact, clones #24 and #27 exhibit a very stable phenotype (see Fig. 1 legend).

Levels of overexpression varied from very mild (#21) to quite impressive; clones #24 and #27 exhibit levels of expression that are higher than those found in MCF7/ADR and 8226/Dox<sub>40</sub>, breast carcinoma and myeloma cells selected with anthracycline that are  $\sim$ 100- and 65-fold resistant to doxorubicin, respectively (Fig. 1 C). Expression in #27 and #24 is comparable to that found for LR73/1-1 cells, which are Chinese hamster ovary fibroblasts transfected with murine MDR 1 and selected with 50 ng/ml vinblastine to  $\sim$ 100-fold resistance to the antimitotic (Gros et al., 1991) and close (within twofold) to the levels found for DC3F-ADX (Biedler and Riehm, 1970), a Chinese hamster lung fibroblast selected to 10,000-fold resistance to actinomycin D (Fig. 1 C). Thus, comparing these data to data in Table I indicates high levels of drug resistance in MDR cells are related more to drug selection than to MDR protein overexpression.

Notably, all hu MDR 1 clones exhibit significant intracellular alkalinization (between 0.21 and 0.35 units) relative to control transfectants (Table I). The present data showing alkalinization in all six independently derived clones, along with a qualitative relationship between alkalinization and MDR protein expression (see DISCUSSION) shows that hu MDR 1 protein overexpression typically promotes intracellular alkalinization in LR73 cells. Since it has been proposed that the acetoxymethyl ester analogues of probes we have used to measure  $\text{pH}_i$  might be “pumped” by the MDR protein (Homolya et al., 1993), we have thoroughly examined this possibility. Fig. 2 presents representative BCECF-AM loading and BCECF washout data for hu MDR 1 and control transfectants and documents that BCECF-AM or BCECF pumping does not occur in these transfectants. Furthermore, calibration curves generated as described in MATERIALS AND METHODS reveal that the  $\text{pH}_i$ -dependent behavior of BCECF is virtually identical in the different cell lines (Fig. 3). That is, even though there are slight differences in the absolute value of 439/490 nm BCECF excitation at various  $\text{pH}_i$ , the shape and slope of the 439/490 nm excitation vs.  $\text{pH}_i$  curves for the different cell lines are indistinguishable. Even if we disregard the data in Fig. 2 and assume BCECF or BCECF-AM is pumped by MDR protein, this pumping function does not compromise our ability to obtain accurate  $\text{pH}_i$ .

#### Examination of $\text{H}^+$ Excretion and $\text{pH}_i/\text{pH}_o$ Relationships with or without $\text{HCO}_3^-$

In culturing drug-selected MDR cells over the years, many investigators have noticed that growth media har-

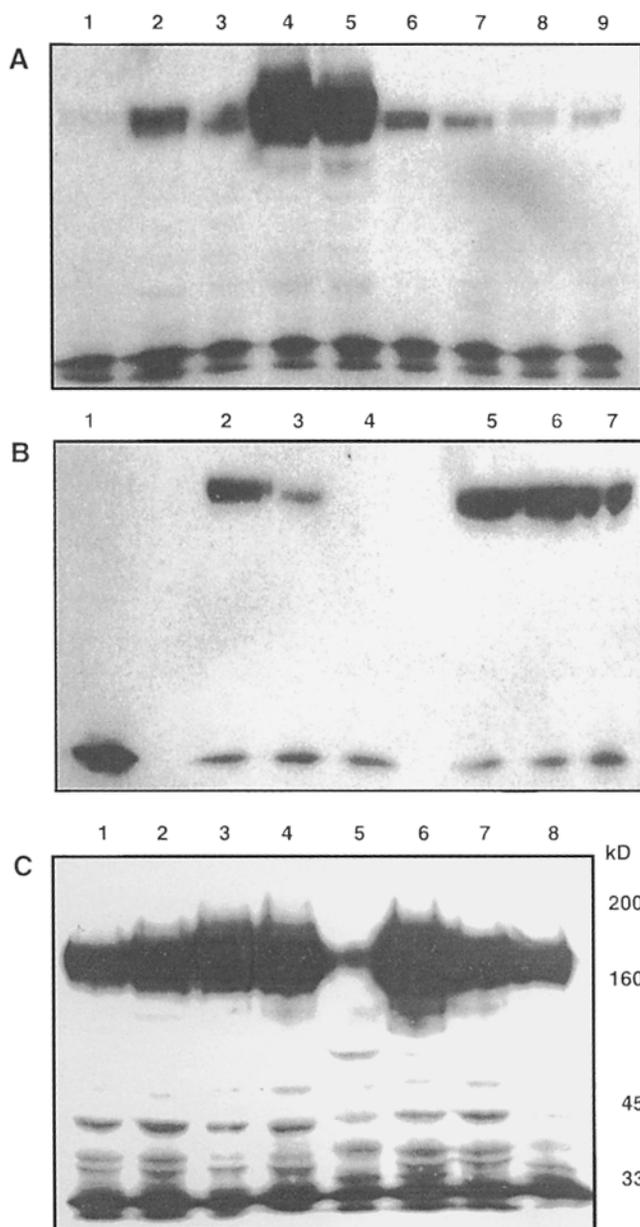


FIGURE 1. Western blot analysis of hu MDR 1 transfectants using the MDR protein-specific mAb C219. The prominent band that migrates at 170 kD is hu MDR 1 protein. In A, B, and C, each lane harbors 100  $\mu$ g total cellular protein, as measured previously (Wei and Roepe, 1994) using BSA as a standard. In A, 8 LR73/hu MDR 1 clones are compared to a negative transfectant control. Lane 1 is a LR73/neo control, and lanes 2-9 are clones #6, 8, 24, 27, 71, 95, 88, and 21, respectively. In B, two clones (#88, #71) are assayed for relative MDR overexpression after multiple passages in the constant presence of G418 only. Lane 1 is a negative transfectant control, lanes 2-4 and 5-7 are the two clones passed 4, 10, and 18 times in the constant presence of G418. Similar loss of MDR protein is seen in other clones (not shown) making it unlikely that loss is chromosomal integration site specific. In the present work, clones #24, 27, and 71 exhibited very stable overexpression (no loss detected within 15 passages). Clones 88, 95, and 21 exhibited stable overexpression for at least 5-6 passages. In C, expression of hu MDR 1 protein in four pure transfectants (including clones #27 and 24, lanes 2 and 3, respectively) is compared to expression of

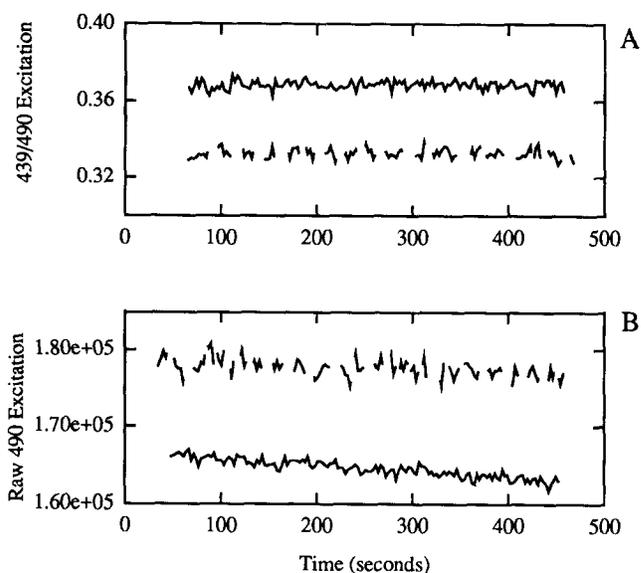


FIGURE 2. Demonstration that the rate of passive leak of BCECF under constant perfusion and the efficiency of BCECF loading (via conversion of BCECF-AM) is virtually identical for hu MDR 1 (dashed line) and control transfectants (solid line) used in the present work. Coverslips were loaded in the presence of BCECF-AM for 30 min as described in MATERIALS AND METHODS, and then placed in the single-cell photometry perfusion chamber. Perfusion with HBSS (37°C, pH 7.33) was initiated, and both 439/490 nm excitation ratios (A) and 490 nm excitation (>530 nm emission) (B) were recorded for single cells. The 439/490 nm trace, which is proportional to  $pH_i$ , (A) is quite stable under constant perfusion during the time course of our experiments. In contrast to previous studies that used selectants (Homolya et al., 1993), we find that MDR and control transfectants convert very similar levels of BCECF-AM (early time, B) in similar time and exhibit virtually identical rates of BCECF leak (B) that varies between 1 and 5% per h. In well over 100 individual experiments, no statistically significant difference in the level of BCECF-AM loading has been observed for these MDR transfectants relative to the parental cells. Along with the observation that the  $K^+$ /nigericin titration curves for the different cell lines are superimposable (see Fig. 3) these data argue that there is no interaction between BCECF or BCECF-AM and the MDR 1 protein.

boring phenol red turns a more yellow color, relative to the media for control drug-sensitive cells, perhaps indicating a greater production of acid by the MDR cells. Since our clones have alkaline  $pH_i$  (Table I), perhaps one explanation for both observations is increased cellular  $H^+$  efflux. We quantified acid production by ratioing DME media absorbance at 560 and 421 nm at dif-

human, hamster, and mouse MDR proteins in MCF7-ADR human breast carcinoma (lane 5) and 8226/Dox<sub>40</sub> human myeloma (lane 8) cells, DC3F-ADX chinese hamster lung (lane 6) cells, and LR73 / 1-1 (lane 7) cells, respectively. Levels of expression in several of our pure transfectants are comparable to the levels recorded for chemotherapeutic drug selected (MCF7-ADR, 8226/Dox<sub>40</sub>, DC3F-ADX) or selectant (LR73/1-1) cell lines, which exhibit particularly high levels of MDR protein overexpression.

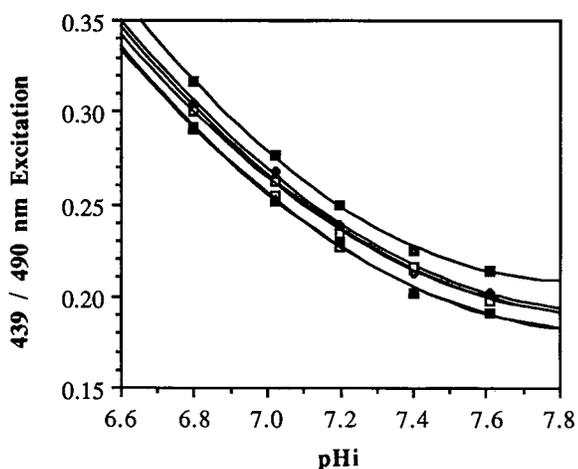


FIGURE 3. Representative calibration solution standard curves for control LR73/neo and the 6 different MDR transfected cell lines summarized in Table I, generated as described in Methods and Luz et al., 1994. Each curve is fit to a 2nd order polynomial,  $R^2 > 0.97$  in each case. Note that behavior of BCECF over the full range of its pH dependent response (pH 5.8–8.0) is sigmoidal in shape and that the quadratic fit (which represents approximately one half of the full titration curve) should not be used to calculate  $\text{pH}_i$  values that lie outside the actual experimental data points. Standard error for each point is  $<6\%$ , but the error bars are omitted for clarity. Note that although absolute values of 439/490 nm excitation at a given  $\text{pH}_i$  may differ for different cell lines (or even different coverslips of the same cell line, data not shown), the shape and slope of the calibration curves are virtually identical, illustrating similar  $\text{pH}_i$ -dependent behavior of trapped BCECF for the different cell lines.

ferent days' growth (Fig. 4). To correct for mild differences in growth rate, data is plotted vs. cell number. The inset to Fig. 4 shows a calibration curve for DME media titrated to different pH. We do not measure significantly increased production of  $\text{H}^+$  for cultures of either clone #24 (Fig. 4, diamonds) or clone #27 (not shown), relative to control cells (Fig. 4, squares). Based on this observation, and estimation of the change in  $\text{pH}_o$  expected for a given increase in the rate of  $\text{H}^+$  efflux, (see legend to Fig. 4), we conclude that alkalinization of the MDR clones is not likely due to an increased rate of  $\text{H}^+$  efflux from these cells.

To further examine  $\text{pH}_i$  regulation, we measured  $\text{pH}_i$  at a variety of  $\text{pH}_o$  while cells were perfused with buffers with or without variable  $[\text{HCO}_3^-]$  (Fig. 5). We have previously observed (Roepe et al., 1993; Luz et al., 1994; Roepe et al., 1994) that cells overexpressing MDR protein exhibit altered  $\text{HCO}_3^-$ -dependent  $\text{pH}_i$  regulation (i.e., reduced  $\text{Cl}^-/\text{HCO}_3^-$  exchanger activity). As shown,  $\text{pH}_i/\text{pH}_o$  relationships obtained in the presence vs. absence of  $\text{HCO}_3^-$  are different for the control cells, whereas they are much more similar for clone #27. These data suggest alkalinization for the MDR clones may be, at least in part, due to perturbation of  $\text{HCO}_3^-$ -dependent mechanism(s).

### Electrical Membrane Potential Alterations

Along with alterations in  $\text{pH}_i$ , importantly, all hu MDR 1 clones were found to be depolarized (Table I). The decrease in  $V_m$  was quantitatively estimated to vary from 8 to 22 mV via  $\text{K}^+$ /valinomycin titration as described (Roepe et al., 1993; Luz et al., 1994).<sup>4</sup>

To further test for differences in membrane potential, we analyzed the rate and steady state of oxocarboxyanine uptake for attached cells under constant perfusion. Members of the  $\text{diOC}_n(3)$  series of oxocarboxyanines differ in their response to membrane potential but only slightly in size (Sims et al., 1974; Wadkins and Houghton, 1995), and not at all in terms of fluorescence excitation/emission wavelengths. Thus, analysis of the relative uptake of a series of the compounds (see MATERIALS AND METHODS) offers a way to analyze  $V_m$  and control for nonspecific effects.

Fig. 6 A–C compares accumulation of  $\text{diOC}_7(3)$ ,  $\text{diOC}_5(3)$ , and  $\text{diOC}_2(3)$ , respectively, for LR73/hu MDR 1 #27 (dashed lines, bottom trace in each comparison) and a control LR73 transfected (solid lines, top trace in each comparison), and Fig. 6 D shows accumulation of the well known  $V_m$  probe  $\text{diOC}_5(3)$  for the series of clones listed in Table I. The inset to Fig. 6 D compares  $\text{diOC}_5(3)$  uptake for control cells in the presence of normal HBSS (top solid line) vs. control cells in the presence of HBSS containing 150 mM  $\text{K}^+$  (high  $\text{K}^+$  HBSS; bottom dashed line). Since high  $[\text{K}^+]_o$  will depolarize the plasma membrane of these LR73 cells (Luz et al., 1994), data in the inset demonstrate the ability of the single-cell method to distinguish between cells exhibiting significantly different  $V_m$ . Clearly, clones exhibiting high levels of MDR protein expression and substantial depolarization via the  $\text{K}^+$ /valinomycin determination (Table I) accumulate oxocarboxyanines that are more responsive to  $V_m$  ( $\text{diOC}_2(3)$  and  $\text{diOC}_5(3)$ , Fig. 6 B and C) at a reduced rate and to a much lower steady state, relative to the control cells, but accumulate  $\text{diOC}_7(3)$  (Fig. 6 A), which does not respond to  $V_m$  very efficiently at these concentrations (see Sims et al., 1974; Wadkins and Houghton, 1995), similarly relative to control cells. Also, with one exception (clone #88) the relative uptake of  $\text{diOC}_5(3)$  for the

<sup>4</sup>Representative  $\text{K}^+$ /valinomycin titration traces for LR73 cells and pure mu MDR 1/LR73 transfectants may be found in Luz et al. (1994); we compensate for the low di-4-ANEPPS signal by averaging at least three traces at each  $[\text{K}^+]$  (see Luz et al., 1994 and *Measurement of Membrane Electrical Potential* in MATERIALS AND METHODS). A double-blind comparison of electrophysiologic and our  $\text{K}^+$ /valinomycin titration methods has recently been published (Wei et al., 1995). Note that all the cell lines examined are derived from a single isolate of the parental LR73 cell line, have very similar size (Table I) and have  $V_m$  that is dominated by  $\text{K}^+$  conductance (not shown, see Roepe et al., 1993). Thus, the most reasonable explanation for substantially different  $\text{K}^+$ /valinomycin null points (Table I) is different  $V_m$ .

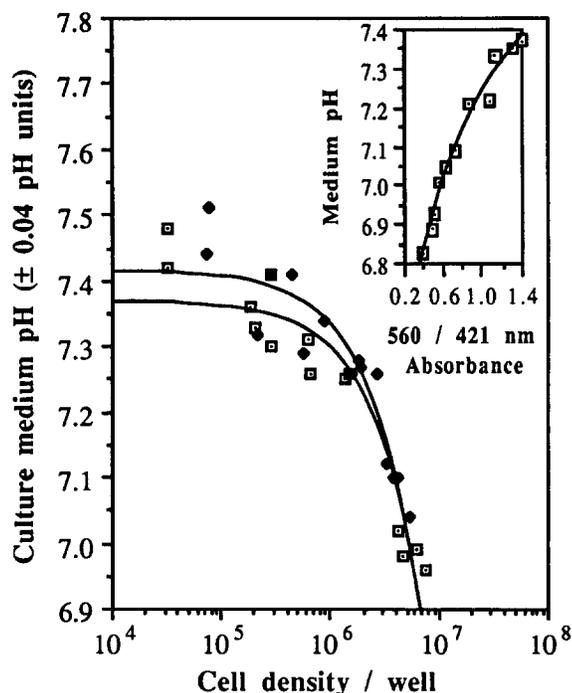


FIGURE 4. Plot of DME culture medium pH vs. cell density for growing cultures of control (*squares*) and MDR 1 (clone #24) transfectants (*diamonds*). Data are fit to exponential equations, ( $R^2 > 0.91$  in each case), and are representative of three separate experiments. The inset shows the change in 560/421 nm absorbance for DME media (equilibrated with 5%  $\text{CO}_2$  and to 37°C) titrated to different pH via addition of 5  $\mu\text{l}$  aliquots of 0.10 N HCl (e.g., addition of 0.50 mM  $\text{H}^+$  between each point). Data is fit to a logarithmic equation,  $R^2 > 0.96$ . From this calibration curve we can conveniently calculate  $\text{pH}_i$  of the culture medium by visible absorption, as well as the medium buffering capacity at various  $\text{pH}_i$ , since known amounts of HCl were added ( $\beta_i = \Delta\text{pH}/\Delta[\text{H}^+]$ ). Even allowing for DME buffering capacity, if MDR protein directly or indirectly translocated  $\text{H}^+$  at a turnover greater than  $\sim 10 \text{ H}^+/\text{MDR protein/s}$ , we should measure a faster acidification for the transfectant culture medium. Using the data in the inset, we calculate the buffering capacity of DME media (between pH 7.33 and 6.93) to be 9.8 mM. We estimate that the minimal pH change that would be reliably detected under these experimental conditions is about 0.05 U/d, and to produce this change the MDR protein present in  $10^6$  cells would need to extrude on the order of  $10^{18} \text{ H}^+/\text{d}$ . Assuming a reasonable site density for MDR protein, we can eliminate models that envision MDR protein directly or indirectly translocates more than  $\sim 10 \text{ H}^+/\text{s}$ . Thus, if MDR protein is (or stimulates) an  $\text{H}^+$  ATPase, or promotes  $\text{H}^+$  efflux some other way, it has very slow turnover at the  $\text{pH}_i$  listed in Table I.

different clones (Fig. 6 D) appears to follow the same trend as  $V_m$  determined by  $\text{K}^+$ /valinomycin titration (Table I). We have no simple explanation for the somewhat unusual accumulation for clone #88, which consistently plateaued slowly, but always remained below accumulation for the controls.

Although the  $\text{diOC}_5(3)$  accumulation method is by no means as quantitative as null point titration, these data are consistent with decreased  $V_m$  for the MDR clones and support the observation that depolarization

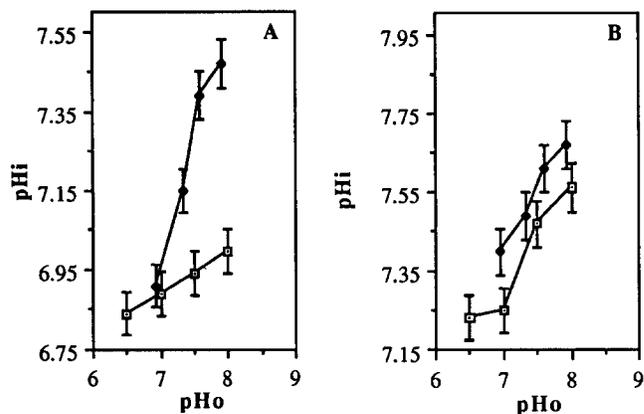


FIGURE 5. Plot of the relationship between  $\text{pH}_i$  and  $\text{pH}_o$  for control (A) and hu MDR 1 clone #27 (B) transfectants. The  $\text{pH}_i$  was measured under constant perfusion by SCP as described (see MATERIALS AND METHODS) and  $\text{pH}_o$  ( $\pm 0.03$  U) was measured for equilibrated perfusate with a pH electrode. Cells were perfused with buffer for 10 min before initiating the measurements. For each coverslip, we either started with high pH perfusate and proceeded to lower pH, or visa versa, collected  $\text{pH}_i$  for 20 cells at each pH (with 10 min equilibration time between buffers) and then computed a standard curve using the same coverslip (see Fig. 3). We noticed slight differences in  $\text{pH}_i/\text{pH}_o$  relationships depending upon whether perfusate went from high to low or low to high pH, but compensated for this slight variation by averaging the results from six different experiments with six different coverslips to compute  $\text{pH}_i$  ( $\pm 0.07$  U) at each  $\text{pH}_o$  ( $\pm 0.03$  U, error bars omitted for clarity). The open symbols represent data collected under  $\text{HCO}_3^-$ -free conditions (perfusion with HEPES buffered saline; 145 mM NaCl, 5 mM KCl, 10 mM glucose, 1.3 mM  $\text{CaCl}_2$ , 0.5 mM  $\text{MgCl}_2$ , 20 mM HEPES adjusted to various pH), and the closed symbols represent data collected during constant perfusion with HBSS adjusted to different pH by adjusting  $\text{HCO}_3^-$  concentration in the medium (at fixed 5%  $\text{CO}_2$ ). The  $\text{HCO}_3^-$  concentrations were 10, 24.2, 50, and 75 mM at pH 6.92, 7.30, 7.57, and 7.93, respectively, and tonicity of the medium was kept constant (relative to normal HBSS) by either addition of 14.2 mM or withdrawal of 25.8 or 50.8 mM NaCl, respectively, along with addition of reduced or increased  $\text{NaHCO}_3$ , respectively (see MATERIALS AND METHODS). Reduction of  $\text{Cl}^-$  concentration by 50 mM for HBSS (via substitution with glutamate) does not in and of itself lead to significant changes in  $\text{pH}_i$  for these cells over the time course of the measurements (not shown).

is related to relative MDR protein overexpression. One could conceivably argue that decreased  $\text{diOC}_2(3)$  and  $\text{diOC}_5(3)$  uptake is due to outward pumping of the compounds by MDR protein, but it would be surprising if MDR protein pumped compounds as structurally diverse as  $\text{diOC}_2(3)$  and vincristine, but not compounds as similar as  $\text{diOC}_7(3)$  and  $\text{diOC}_5(3)$ . Considering all the data together, decreased carbocyanine uptake due to lower  $V_m$  is a much more logical explanation.

#### Resistance of the Clones to Chemotherapeutic Drugs and Chemoreversal

The clones were also analyzed for resistance to the chemotherapeutic drugs doxorubicin (Dox), vincristine (Vncr), and colchicine (Col) (Table I). As previously

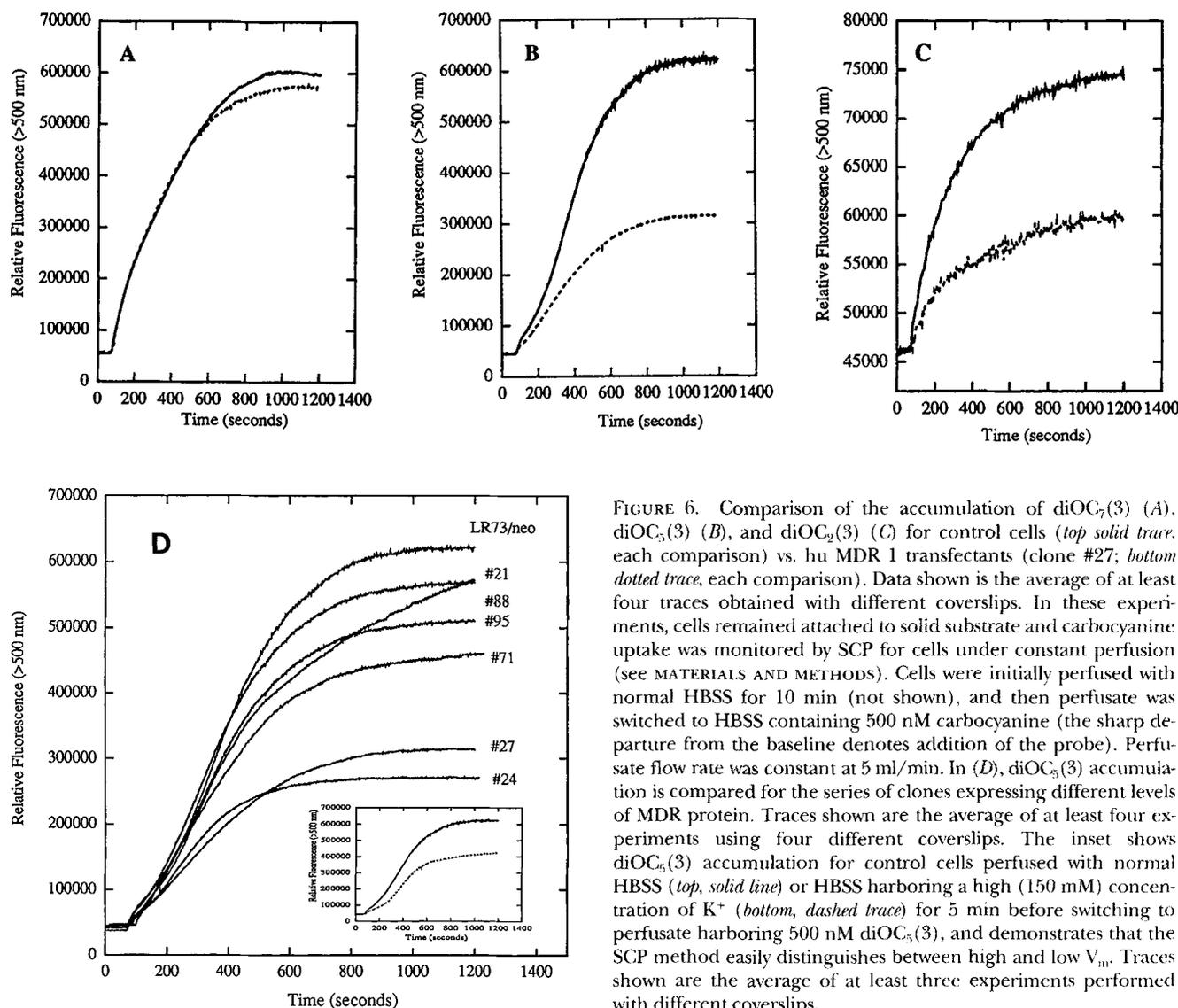


FIGURE 6. Comparison of the accumulation of diOC<sub>7</sub>(3) (A), diOC<sub>5</sub>(3) (B), and diOC<sub>2</sub>(3) (C) for control cells (top solid trace, each comparison) vs. hu MDR 1 transfectants (clone #27; bottom dotted trace, each comparison). Data shown is the average of at least four traces obtained with different coverslips. In these experiments, cells remained attached to solid substrate and carbocyanine uptake was monitored by SCP for cells under constant perfusion (see MATERIALS AND METHODS). Cells were initially perfused with normal HBSS for 10 min (not shown), and then perfusate was switched to HBSS containing 500 nM carbocyanine (the sharp departure from the baseline denotes addition of the probe). Perfusate flow rate was constant at 5 ml/min. In (D), diOC<sub>5</sub>(3) accumulation is compared for the series of clones expressing different levels of MDR protein. Traces shown are the average of at least four experiments using four different coverslips. The inset shows diOC<sub>5</sub>(3) accumulation for control cells perfused with normal HBSS (top, solid line) or HBSS harboring a high (150 mM) concentration of K<sup>+</sup> (bottom, dashed trace) for 5 min before switching to perfusate harboring 500 nM diOC<sub>5</sub>(3), and demonstrates that the SCP method easily distinguishes between high and low V<sub>max</sub>. Traces shown are the average of at least three experiments performed with different coverslips.

observed for 3T3/ $\mu$  MDR 1 infectants (Guild et al., 1988) or mass populations of pure LR73/ $\mu$  MDR 1 transfectants (Devault and Gros, 1990), drug resistance exhibited by these pure hu MDR 1 transfectants is low compared to MDR 1 overexpressing cell lines that are selected or maintained with chemotherapeutic drugs (see INTRODUCTION and Fig. 1 C). Thus, data in Table I and Fig. 1 C indicate that exposure to chemotherapeutic drugs must significantly perturb the resistance profile unequivocally attributable to MDR protein overexpression.

To test this idea further, clone #27 was grown in the presence of either doxorubicin (cell line 27D500), vincristine (27V500), or colchicine (27C500) over a period of several weeks to generate three derivative cell lines (see Table II legend). These three "selectants" were then assayed for drug resistance and for relative

MDR protein expression as described in Fig. 1 (Table II). Interestingly, growth in the presence of either doxorubicin or colchicine not only increased resistance to the "selecting agent," as would be anticipated, but increased resistance to the other two drugs as well. In contrast, growth on vincristine did not increase resistance to the other drugs as significantly. Relative expression of MDR protein increased only slightly (10–30%) for the selectants relative to the transfectant (Table II). For each selectant, the "resistance profile" (rank of preferential resistance to various drugs) changes. For example, 27C500 was similarly resistant to the three drugs, whereas 27D500 was clearly more resistant to Dox, and clone #27 grown in the absence of chemotherapeutic drug more resistant to Vncr.

Studies have shown that several compounds, including the channel blocker verapamil and the immuno-

TABLE II  
Relative Resistance of Selectants

Cell line	Fold resistance to			Relative MDR protein
	Dox	Vncr	Col	
LR73/#27	2.7	9.5	3.2	9.7
27D500	21.1	15.3	10.2	12.2
27V500	6.0	13.2	3.5	11.3
27C500	12.2	11.5	9.6	11.2

Fold resistance to doxorubicin (dox), vincristine (vncr); and colchicine (col) for three drug exposed derivatives ("selectants") of clone #27, as well as relative MDR protein expression (relative to clone #21, see Table II). The three selectants were produced by growing clone #27 cells in the presence of either 150 nM Dox, Vncr, or Col for 2 wk, and then 3 wk more in the presence of 500 nM respective drug. These "drug conditioning" conditions should be distinguished from formal selection with chemotherapeutic drug. Resistance was analyzed by growth inhibition assays (see MATERIALS AND METHODS) and relative survivability at 6 concentrations of drug was analyzed in triplicate (see Wei and Roepe, 1994; SE for each point <5%). After fitting survivability curves (e.g. as shown in Fig. 7) to an exponential (Wei et al., 1995; each fit  $R^2 < 0.92$ )  $IC_{50}$  for a given drug was calculated, and ratioed vs.  $IC_{50}$  determined for LR73/neo to compute fold resistance. Relative MDR protein expression is the average of two densitometric scans from a Western blot performed as described in Methods; both LR73 #27 and LR73/neo were also resolved on this gel as an internal calibration.

suppressive agent cyclosporin A, act as chemoreversal agents, meaning they reverse the drug resistance exhibited by MDR cells (Ford and Hait, 1990). Reversal of resistance at nontoxic doses of chemoreversal agent is not typically complete, and the level of drug resistance mediated by MDR protein is low (Table I). Also, these compounds compete with chemotherapeutic drugs for nonspecific membrane association (Wadkins and Houghton, 1993), which might confer chemosensitizing ability independent of MDR protein. For these reasons it is unclear whether chemoreversal agents reverse MDR protein-mediated MDR. Therefore, we tested whether the drug resistance in our transfectants was reversed by verapamil and cyclosporin A. Fig. 7 demonstrates that, indeed, these compounds resensitize LR73/hu MDR 1 #27 to the toxic effects of vincristine. Similar results were obtained for two other clones exhibiting similar levels of hu MDR 1 protein overexpression (data not shown). However, importantly, cyclosporin A was found to be toxic above 2  $\mu$ M, thus apparent complete "reversal" at 2.5  $\mu$ M is due to a combination of reversal effects and cyclosporin toxicity. Similarly, we could not demonstrate complete reversal with nontoxic levels of verapamil (<10  $\mu$ M).

*Drug Resistance for Fibroblasts with Alkaline  $pH_i$  or Exposed to High  $[K^+]_o$*

In testing the  $pH_i/V_m$  altered partitioning model, it is not only critical to determine how much drug resistance is explicitly due to overexpression of MDR pro-

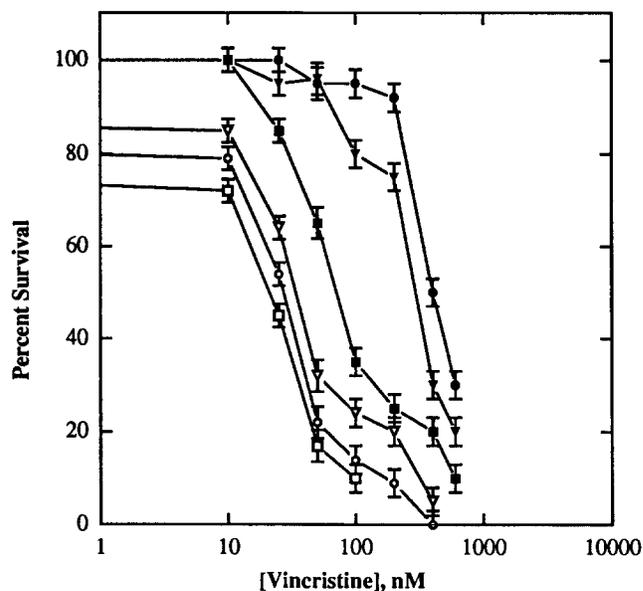


FIGURE 7. Relative survivability via growth inhibition (see MATERIALS AND METHODS) vs. increasing concentrations of vincristine for clone #27 (solid circles) or control transfectants (open circles) in the absence of modulators, or clone #27 analyzed in the presence of either 1  $\mu$ M (open triangles) or 2.5  $\mu$ M (open squares) cyclosporin A, or 1  $\mu$ M (closed triangles) or 5  $\mu$ M (closed squares) verapamil. Very similar data was also obtained for clone #24 (not shown). Nearly complete "chemoreversal" is seen at nontoxic levels (1  $\mu$ M) of cyclosporin A, but sensitization to an extent greater than the transfectant control is seen at 2.5  $\mu$ M due to toxicity of cyclosporin A at this concentration (hence open squares to the left of the curve for the control). 5  $\mu$ M verapamil provides for 60–70% reversal of the resistance due to MDR 1 protein overexpression.

tein, but also whether the extent of alkalization and/or depolarization observed is sufficient to lead to the measured drug resistance. Since drug resistance is typically assayed over days in cell culture, but most manipulations of  $pH_i$  and  $V_m$  are only reliable for minutes/hours (or are toxic thereafter) this is challenging. Analysis of CFTR clones that showed depolarization is sufficient to cause low-level MDR has recently been reported (Wei et al., 1995). To test whether elevated  $pH_i$  is sufficient to cause MDR, and to further test the effects of depolarization, we devised a short exposure cytotoxicity assay (see MATERIALS AND METHODS) for control cells not expressing MDR protein but elevated in  $pH_i$  via growth at different percentages of  $CO_2$ , or depolarized by exposure to high  $[K^+]_o$  (see Fig. 6 D, inset).

Fig. 8 presents  $pH_i$  data for LR73 cells grown for 48 h at 5%  $CO_2$ , followed by 72 h at 10%  $CO_2$ , and then 72 h more at 5%  $CO_2$ . Initial exposure of LR73 cell cultures to higher percentages of  $CO_2$  (at fixed  $^-HCO_3$  in the medium) leads to fast acidification of  $pH_o$ , as reported (Wei and Roepe, 1994) and concomitant, but not as pronounced, acidification of  $pH_i$  (Fig. 8). After  $pH_i$  plateaus during high percent  $CO_2$  "conditioning," and

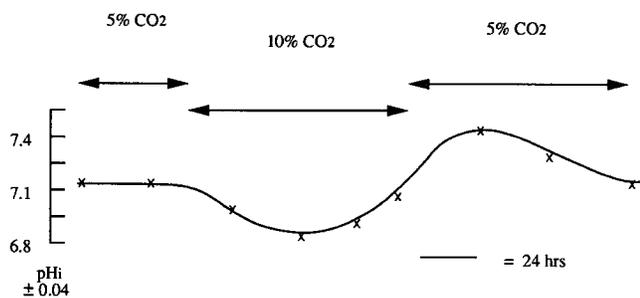


FIGURE 8. Alterations in  $pH_i$  for LR73 cells upon pulsing growing cells with different percentages of  $CO_2$ . Cells were grown as described in methods,  $pH_i$  was measured for these cells approximately every 24 h in triplicate (at the  $pH_o$  of the media under these conditions, see Wei and Roepe [1994]), and the data averaged (the X shown in this plot  $\pm 0.04$  pH U). We note a small yet reproducible transient elevation in  $pH_i$  for cells at 5%  $CO_2$  after they are stimulated to presumably increase the rate of  $H^+$  removal via growth under acidic (10%  $CO_2$ ) conditions. Dramatic overexposure of Western and Northern blots reveals that no induction of MDR protein or mRNA expression occurs under these conditions (not shown, see Wei and Roepe, 1994).

assuming the density of the culture is kept subconfluent, the cells “readjust” their  $pH_i$  homeostasis and nearly recover normal  $pH_i$ . When these conditioned cells are suddenly transferred back to 5%  $CO_2$  and fresh, pre-equilibrated media is added,  $pH_i$  “overshoots” for 12–24 h, and then re-normalizes once again. Thus, a window of alkaline  $pH_i$  is available for a short period of time in the absence of MDR protein overexpression.

To test whether these alkaline cells were drug resistant, we performed short exposure cytotoxicity assays (see MATERIALS AND METHODS) (Fig. 9, A–C). Overexposure of Western and Northern blots revealed no increased MDR protein or mRNA overexpression in the cells after manipulating percentage of  $CO_2$  (data not

shown, see Wei and Roepe, 1994). Nonetheless, cells elevated in  $pH_i$  by 0.16–0.24 units did exhibit low, but measurable, resistance to doxorubicin, vincristine, and colchicine. Resistance to doxorubicin and vincristine was higher (approximately four- to fivefold and three- to fourfold, respectively) relative to colchicine resistance (approximately twofold).

The fold-resistance exhibited by these alkaline cells is slightly lower (on average) than the resistance of the two clones exhibiting particularly high levels of hu MDR 1 protein expression (clones #24 and 27, see Table I) yet is comparable to the resistance exhibited by the lower-level expressors. However, we do not measure significant alterations in  $V_m$  for  $CO_2$  pulsed cells (data not shown), and a variety of data (reviewed in Roepe, 1995) suggest both decreased  $V_m$  and elevated  $pH_i$  contribute to MDR protein-mediated drug resistance. Thus, one key element of the MDR phenotype is absent for these  $CO_2$  pulsed cells. Also, due to the nature of the assay (see MATERIALS AND METHODS) these levels of resistance may be underestimates.

Similar cytotoxicity assays were performed for LR73 cells resuspended in DME media containing different  $[K^+]_o$  (Fig. 10). LR73 cells are depolarized (cf., Fig. 6 D, inset) in media containing 15 mM  $Na^+$ /127 mM  $K^+$  (dashed lines, Fig. 10). Fig. 10 shows these depolarized cells (estimated 25 mV  $V_m$  by comparing data in Table I and Fig. 6 to similar diOC<sub>5</sub>(3) data for cells exposed to high  $K^+$  DME for similar time, not shown) are also resistant to vincristine (three- to fourfold), doxorubicin (three- to fourfold), and colchicine (~1.5-fold). The resistance is not due to  $pH_i$  or  $V_i$  changes secondary to treatment with high  $[K^+]_o$  (see legend to Fig. 10). These data agree with other studies (Wei et al., 1995) that measured mild drug resistance for stably depolarized 3T3/CFTR clones.

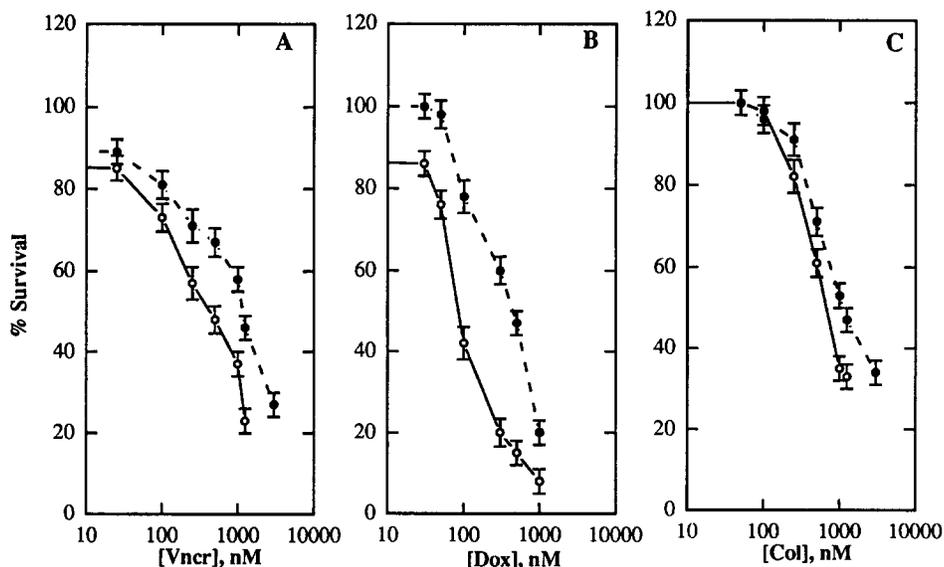


FIGURE 9. Relative resistance to Vncr (A), Dox (B), and Col (C) for cells pulse elevated in  $pH_i$  as described in Fig. 8. In these experiments, cells were harvested either before  $CO_2$  pulse treatment (open symbols, solid lines) or at the peak of  $pH_i$  elevation (~12–15 h after shifting back to 5%  $CO_2$ ). They were washed and gently tumbled in eppendorf tubes with various concentrations of drug as described in MATERIALS AND METHODS. Cells were then stained after 3 d growth at 5%  $CO_2$ . The results from 4 such assays (16 determinations at each drug concentration) were then averaged and are shown  $\pm$  SE.

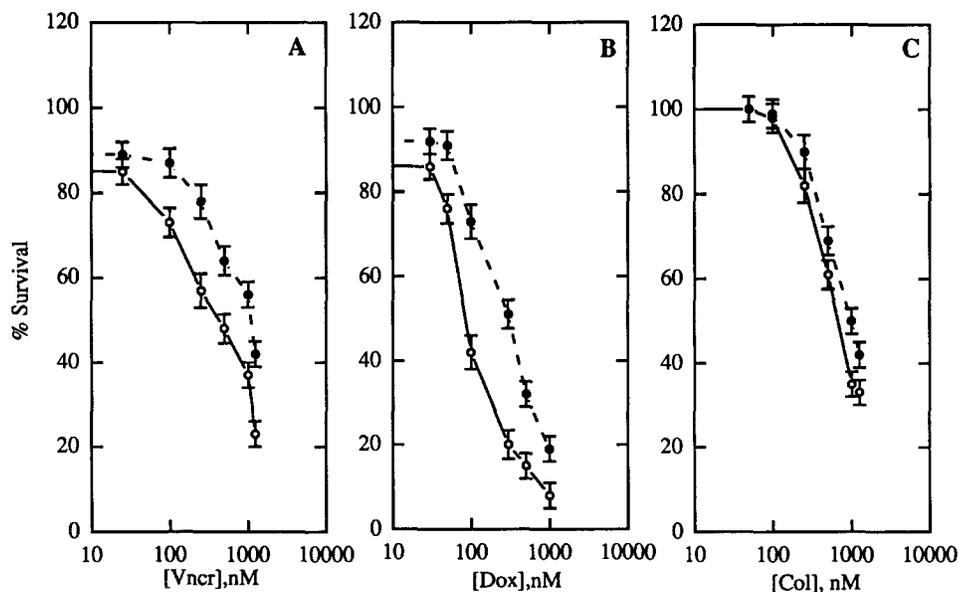


FIGURE 10. Relative resistance to Vncr (A), Dox (B), and Col (C) for LR73 cells depolarized by suspension in medium containing 127 mM  $K^+$  (see Fig. 6 D, *inset*). In these experiments, cells were harvested, washed in fresh DME media containing either 127 mM  $Na^+$  and 15 mM  $K^+$  (*solid lines*) or 127 mM  $K^+$  and 15 mM  $Na^+$  (*dashed lines*) and resuspended in the same media plus various concentrations of drug (see MATERIALS AND METHODS). The results from 3 assays (12 determinations at each drug concentration) were then averaged and are shown  $\pm$ SE. As demonstrated elsewhere (Roepe et al., 1994) the presence of high  $[K^+]_o$  for LR73 cells does not change steady state  $pH_i$ , substantially, and we do not measure significantly different  $V_i$  for these high  $[K^+]_o$ -treated LR73 (data not shown), thus the most straightforward interpretation is that mild drug resistance in these cells is due to membrane depolarization.

#### Drug Accumulation

Another feature of the MDR phenotype is altered chemotherapeutic drug accumulation. We measured the accumulation of  $^3H$  derivatives (see MATERIALS AND METHODS) of anthracycline (Fig. 11, *open bars*), vinca alkaloid (*dotted bars*), and colchicine (*solid bars*) for two transfectants exhibiting either high (#27) or low (#95) levels of MDR protein overexpression (see Table I) and compared this accumulation to that exhibited by control LR73/neo transfectants pulse elevated in  $pH_i$  by the  $CO_2$  pulse method or depolarized in high  $[K^+]$  medium as described above (Fig. 11). The two- to three-fold levels of drug resistance are essentially mirrored by about twofold lower drug accumulation as measured under these conditions (Fig. 11, *open and solid bars*). In contrast, relative accumulation of  $^3H$  vinca is more markedly reduced for clone #27 (to  $\sim 6\%$  of the level seen in the controls), which is consistent with the comparatively high level of vinca resistance for this cell line ( $\sim 10$ -fold) shown in Table I.

We emphasize that one should not expect a linear or simple correspondence between fold drug accumulation and fold drug resistance. Even if cells accumulate very similar levels of a weakly basic drug, if that drug is compartmentalized inside the cell in a different fashion (due to, for example, different  $\Delta pH$  between cytosolic and vacuolar milieu), the accessibility of the drug to its target may be different, further affecting resistance. Nonetheless, a few general expectations are veri-

fied by these results, including that depolarization does not appear to alter accumulation of the uncharged drug colchicine as dramatically as it does the other compounds, although it does appear to have a mild effect (see also Wei et al., 1995).

#### DISCUSSION

##### *Which Cellular Alterations Are Unequivocally due to MDR Protein Overexpression?*

Although many studies have previously noted elevated  $pH_i$  for MDR cells (reviewed in Roepe, 1995), there has been at least one contrasting observation for a chemotherapeutic drug-selected cell line (Altenberg et al., 1993) in a study that used different techniques, relative to most other work.<sup>5</sup> Along with elevated  $pH_i$ , several other important biophysical characteristics of MDR cells have been reported, including decreased  $V_m$  (Roepe et al., 1993), altered  $Cl^-$  transport (Valverde et al., 1992), altered membrane lipid composition (Ramu et al., 1984), and increased vesicular traffic (Sehested et al., 1987). It is not difficult to envision how these characteristics might contribute to drug resistance via

<sup>5</sup>Altenberg et al. (1993) analyzed  $pH_i$  for populations of 100–150 cells in an atmosphere of 95%  $O_2$  after loading the cells with BCECF-AM for relatively long time (60 min) in the presence of detergent (the pluronic F-127), techniques which are not followed in other work (Luz et al., 1994; Roepe et al., 1994; Simon et al., 1994; and this paper).

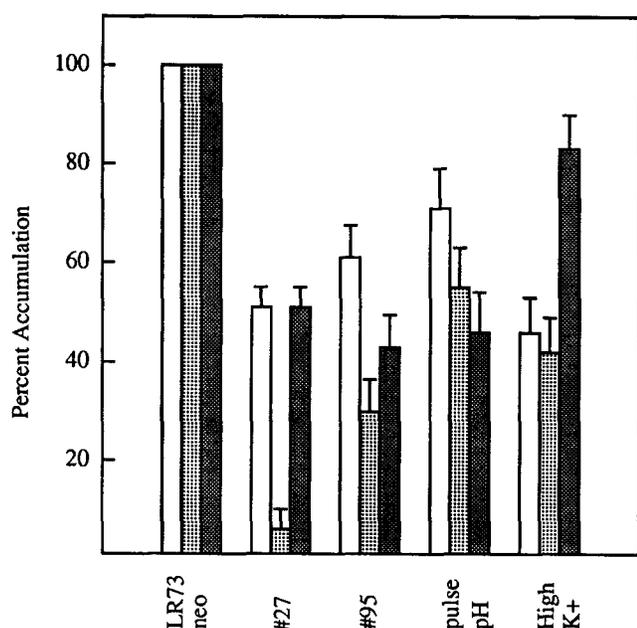


FIGURE 11. Relative percent accumulation of <sup>3</sup>H analogues of representative chemotherapeutics (daunorubicin [open bars], vinblastine [dotted bars] and colchicine [solid bars], see MATERIALS AND METHODS) for (from left to right) control LR73/neo transfectants without MDR protein expression, clone #27, clone #95, control transfectants pulse elevated in pH<sub>i</sub> by 0.21 U as described in Fig. 8, and control transfectants depolarized 25 mV by incubation in DME medium containing 127 mM K<sup>+</sup> (see MATERIALS AND METHODS) as described in Fig. 10. Daunorubicin and vinblastine were available as less expensive <sup>3</sup>H analogues (New England Nuclear and Amersham, respectively). Differences in cell resistance to daunorubicin vs. doxorubicin (which differ by a single OH group) or to vincristine vs. vinblastine (which differ by a single oxygen on the catharanthine group) are not statistically different for the cell lines described (Wei, L.Y., and Roepe, P.D., unpublished data, and see RESULTS). The most profound alteration in accumulation is for clone #27, which accumulates about 6% of vinca alkaloid, relative to controls.

altering diffusion or partitioning of hydrophobic drugs (Roepe, 1995), thus it is tempting to conclude that they may be directly or indirectly due to MDR protein overexpression. However, as is the case with elevated pH<sub>i</sub>, a few contrasting observations with respect to these phenotypic features have also been reported (e.g., Ehring et al., 1994; Callaghan et al., 1992). Therefore, it has been argued that these features might be epiphenomena unrelated to MDR protein overexpression.

In analyzing experiments with MDR cells, it must be emphasized that chemotherapeutic drugs are very powerful compounds. They are not only toxic, but most are mutagenic or even teratogenic, and in certain situations they induce differentiation and other complex phenomena. For example, anthracyclines like doxorubicin form reactive semiquinones and aglycones upon one and two electron reduction, form complexes with iron and other ions, significantly perturb the physical characteristics of membranes, and may even alter criti-

cal signal transduction. They alkylate DNA, poison mitochondria, inhibit topoisomerases and other enzymes, and directly modulate second messenger levels, among having other effects. As analysis of MDR protein function has become more detailed, it has therefore become increasingly difficult to separate effects due to MDR protein overexpression from other effects due to drug exposure, and this has dramatically confused interpretation. Although heterologous expression of MDR proteins (e.g., in yeast or bacteria) offers one avenue for improvement, these systems obviously do not accurately model tumor cells, thus important pharmacologic issues cannot be explored. Also, in contrast to some current practices, data collected with heterologous systems need to be interpreted based on expectations that come from the study of pure transfectants, not drug selected MDR cells.

#### Construction of "True" MDR Transfectants

Therefore, it is crucial that phenotypic alterations unequivocally due to MDR protein overexpression be precisely defined. As described, one convenient way to do this is to study pure MDR 1 transfectants that are not selected or maintained on chemotherapeutic drugs. We have successfully engineered a series of these pure transfectants exhibiting different levels of MDR protein overexpression. Surprisingly, the level of drug resistance exhibited by the clones is much lower than might be expected based on the behavior of drug-selected MDR cells. Our data show that exposure to chemotherapeutic drugs is more important for conferring high (>10-fold) levels of resistance than the MDR protein overexpression that might accompany this exposure. This point is important when considering how extensively MDR protein might alter cellular drug accumulation. That is, a common misconception based on high levels of resistance for drug selected MDR cells is that MDR protein overexpression must somehow lower intracellular levels of drugs by a much larger extent than is the case (Fig. 11). Reduced accumulation due to MDR protein can be easily explained by V<sub>m</sub> and pH<sub>i</sub> perturbations (Wadkins and Roepe, 1996), and does not require that we invoke a drug pump model (Gottesman and Pastan, 1993; Higgins and Gottesman, 1992).

Many laboratories have had difficulty constructing pure transfectants for reasons that remain vague, but that might be a consequence of relatively poor chromosomal integration of the very large MDR 1 cDNA, or perhaps deleterious effects of the protein. The latter is suggested due to the loss of MDR protein expression for some clones passaged multiple (one to two dozen) times. We could not unequivocally conclude if this was a random or directed genetic event, but clonal selection and the frequency of this phenomenon (Hoffman, M.M., and P.D. Roepe, unpublished data) suggest the

later is formally possible. However, this phenomenon could also be due to the cDNA integration context for particular clones. The fact that clones #24 and #27 (as well as several other high-level expressors recently isolated, data not shown) exhibit a stable phenotype argues against a strong selective advantage for low expression under our culture conditions. In any case, since we have isolated several independent high-level overexpressing clones that exhibit a similar and stable phenotype (Table I) at a low but reasonable frequency (see MATERIALS AND METHODS), it is unlikely that we preferentially isolate clones expressing randomly generated MDR 1 mutants.

#### *Characteristics of the Clones and Implications for Multidrug Resistance*

These clones exhibit: (a) elevated  $pH_i$  and altered  $pH_i$  regulation, (b) lowered  $V_m$ , (c) low levels of drug resistance that can be explained by the changes in  $pH_i$  and/or  $V_m$ , and (d) partial reversal of drug resistance by nontoxic levels of verapamil and cyclosporin A.<sup>6</sup>

Trends in alkalinization and depolarization are apparent upon plotting these parameters vs. MDR protein expression (Fig. 12); however, analysis of more clones is required before these trends should be considered universal. When the sum of  $V_m$  and  $\Delta pH$  is plotted vs. relative MDR protein overexpression (Fig. 12 C), a nearly flat line is obtained, as also reported for drug selected myeloma derivatives (Roepe et al., 1993), indicating the decrease in  $V_m$  nearly mirrors the increase in  $\Delta pH$ . Importantly, however, since different cells have different mechanisms and priorities for regulating  $pH_i$ ,  $V_m$ , and volume, different degrees of the changes we have found are likely for different cell types (e.g., cells with high vs. low  $Na^+/H^+$  exchanger activity, or AE 1 vs. AE 2 isoform expression) engineered to overexpress MDR protein.

By comparing Table I to Figs. 8, 9, 10, and 11 and to data recently published elsewhere (Wei et al., 1995), we propose that changes in  $V_m$  and  $pH_i$  for the MDR transfectants are sufficient to confer the levels of drug resistance due to MDR protein overexpression. It is unnecessary to invoke a nonspecific drug pump model to explain the MDR protein-mediated MDR phenotype. The  $pH_i/V_m$  altered partitioning model avoids the significant kinetic, thermodynamic, and specificity difficulties inherent in the drug pump model (Roepe et al.,

1996; Wadkins and Roepe, 1996). However, several important questions remain. For example, we do not yet know whether  $pH_i$  and  $V_m$  effects are additive or synergistic with respect to drug resistance. It is reasonable to suggest that changes in cytosolic  $pH_i$  might perturb subcellular distribution of some weakly basic drugs since the  $\Delta pH$  across compartmental membranes will also be altered. This effect and others likely have additional impact upon drug partitioning and resistance (see Roepe, 1995; Wadkins and Roepe, 1996). The sole explanation for drug resistance due to changes in  $pH_i$  and  $V_m$  is likely not simplified altered equilibrium transmembraneous distribution (e.g., weak base partitioning or altered equilibrium partitioning of cations due to altered  $V_m$ ). In contrast, MDR is likely due to the sum of several mechanisms acting together. Due to the complexity of chemotherapeutic drug action, which includes cytotoxic and growth inhibitory functions as well as the ability to induce programmed cell death, or apoptosis (interestingly, an apparently  $pH_i$ -dependent process, see Li and Eastman, 1995; Pérez-Sala et al., 1995), we do not necessarily expect simple relationships between drug transport and drug resistance.

Exposure of our cell lines to different chemotherapeutic drugs alters the level of drug resistance and the resistance profile. These results have important implications for studies wherein mutant MDR cDNAs are transfected into cells that are then subsequently grown in the presence of drugs under different conditions relative to cells transfected with wild-type cDNA (see Choi et al., 1988; Gros et al., 1991). These data have almost always been interpreted to mean that the mutant MDR proteins must pump various drugs with different efficiencies, relative to the wild type, because the drug binding site has been mutated. Alternatively, perhaps the data indicate other (non-MDR protein-mediated) drug resistance mechanisms have been induced with different efficiencies upon the different drug-selection conditions.

#### *Possible Complexities in Interpretation of the Present Data*

Since much of our analysis depends upon intracellular probes and since the MDR phenotype is characterized by altered cellular retention of compounds, we are concerned about possible complexities in interpretation. Some investigators propose that fluorometric probes of  $pH_i$  and  $V_m$  are pumped by the MDR protein and that this pumping function could alter our conclusions. Several arguments suggest this is not the case: (a) Our measurements of loading and leak of BCECF-AM and BCECF, respectively, are in contrast to previous work with drug selected cells (Homolya et al., 1993). Our data argue that AM derivatives are not pumped by MDR protein and that previous observations that led to this conclusion may be due to drug selection or other

<sup>6</sup>The molecular effects of reversal agents are complicated (see Wadkins and Houghton, 1993), and we will address several of these issues elsewhere (Wei, L.Y., and P.D. Roepe, manuscript in preparation). Interestingly, however, Keizer and Joenje (1989) as well as our own laboratory (Roepe, 1992) previously reported that exposure to verapamil lowered elevated  $pH_i$  for MDR cell lines, and the Gupta laboratory (Vayuvegula et al., 1988) previously reported that either verapamil or cyclosporin A repolarized MDR cells.

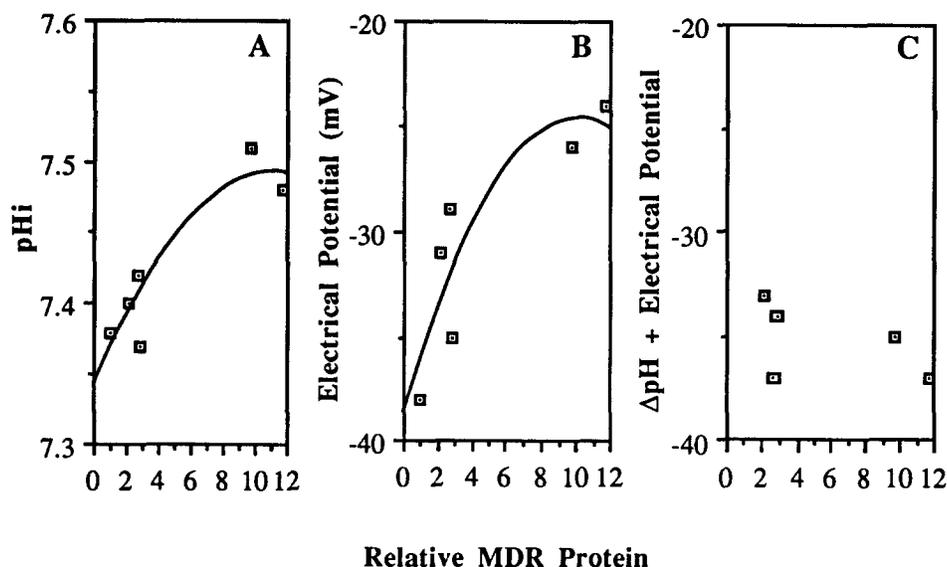


FIGURE 12. Relationship between relative MDR protein expression in the series of transfectants and (A)  $pH_i$ , (B)  $V_m$  (from Table I), and (C) the mathematical sum of  $pH$  and  $V_m$  (in mV, following the convention that alkaline inside is negative). In A and B the data are fit by a 2nd order polynomial,  $R^2 > 0.82$  in each case.

factors. Even if the compound was somehow pumped, the fact that the different standard curves have similar slope (see Fig. 3) shows our calibration in different cell lines is reliable. Via similar arguments, ratiometric measurements with ANEPPS are reliable. (b) Previous analysis of the rate of carbocyanine efflux from MDR vs. sensitive cells showed that there was essentially no difference (Roepe et al., 1993); if these compounds were pumped out of the cell by MDR protein there should be an increased rate of efflux (see Roepe, 1992). (c) Different members of the diOC<sub>n</sub>(3) series are very similar in chemical structure; much more similar than, for example, vincristine and doxorubicin which, via the pump hypothesis, are both substrates for the pump. Although we cannot completely disprove the notion, it is thus difficult to envision why the putative pump would recognize vincristine, doxorubicin, and diOC<sub>5</sub>(3), but not diOC<sub>7</sub>(3). A simpler explanation, also consistent with other data (see Roepe et al., 1993; Luz et al., 1994; Roepe, 1995; Table I), is that the MDR clones have lower  $V_m$ .

#### Future Implications

These data strongly support the altered partitioning model for MDR protein function (Roepe, 1995; Wadkins and Roepe, 1996), resolve several unsettled pharmacologic issues in the MDR literature, and have important implications for the continued evolution of chemotherapy. Consideration of physical-chemical factors in describing the pharmacology of drugs is almost as old as pharmacology itself; however, appreciation for the extent and manner in which cells and microorganisms can alter physical-chemical effects in the course of developing drug resistance is less well appreciated. Examination of the literature pertaining to other exam-

ples of "ABC-MDR" (i.e., overexpression or mutation of a member of the ABC family thereby conferring pleiotropic drug resistance), for example, pfMDR1-mediated chloroquine resistance in malarial parasites perhaps being accompanied by altered  $\Delta pH$  for digestive vacuoles, multidrug resistance-related protein-mediated drug resistance in lung carcinoma perhaps being accompanied by alterations in  $Cl^-$  and  $K^+$  transport, or the apparent role of  $V_m$  in the CFTR-mediated MDR phenotype (Wei et al., 1995), leads us to suggest two important possibilities: (a) a general mechanism for how ABC transporters mediate drug resistance may exist through the proteins regulating  $V_m$ ,  $pH_i$ , or volume, and (b) other undiscovered pathways to drug resistance may also involve  $V_m/pH_i$  perturbations, but via different molecular mechanisms. Indeed, it is interesting to note that the *B. subtilis tet B(L)* locus (which confers tetracycline resistance) encodes a  $Na^+/H^+$  antiporter (Cheng et al., 1994) and that the sapABCDF complex, which confers resistance to toxic peptides and other compounds in *S. typhimurium*, regulates  $K^+$  channel activity (Parra-Lopez et al., 1994).

We suggest that a variety of phenomena reported on in the MDR literature should be re-examined with cell lines similar to those used in the present work to determine whether they are unequivocally due to MDR protein overexpression. This point of view is reinforced by considering the levels of drug resistance exhibited by pure MDR transfectants vs. selectants and drug-selected cells; in most reports it is not emphasized that these differ greatly. Also, even if MDR protein overexpression is an important event in the development of a MDR phenotype, other events that occur during continued drug selection likely complicate matters. These might even be important targets for therapy.

Based on this work and other analysis (Roepe, 1995; Roepe et al., 1996; Wadkins and Roepe, 1996), we feel the altered partitioning model for MDR protein is currently much more reasonable, even if it does conflict

with the very popular drug pump model. Its valuable predictive abilities (see also Wei and Roepe, 1994; Wei et al., 1995) should be examined further.

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We thank our colleagues Taotao Ling, Subham Basu, and Dr. Friederike Fritz for measuring steady state pH<sub>i</sub> vs. pH<sub>o</sub> for LR73/hu MDR 1 clone #27, help with tissue culture, and helpful discussions, respectively. We also thank Dr. Kathy Scotto (Sloan-Kettering Institute, New York, NY) for hu MDR 1 cDNA, Dr. Phillippe Gros (McGill University, Montreal, Canada) for EX4N7 cells, Dr. Olaf Andersen (Cornell University Medical College) for very important suggestions, and Drs. Kathy Scotto (Sloan-Kettering Institute) and Randy Wadkins (Naval Research Laboratories) for additional helpful discussions. We are indebted to Dr. David Gadsby (The Rockefeller University) for encouraging us to continue on in spite of it all.

This work was performed in the Raymond and Beverly Sackler Foundation laboratory at the Sloan-Kettering Institute and was supported by the Wendy Will Case Fund, the Cystic Fibrosis Foundation, the National Institutes of Health (RO1 GM54516 and GM55349), and a Cancer Center Support Grant (NCI-P30-CA-08748). P.D. Roepe is a Sackler Scholar at Memorial Sloan-Kettering Cancer Center.

*Original version received 13 December 1995 and accepted version received 15 July 1996.*

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